

Human Immunodeficiency Virus Type-1 mRNA Splicing Pattern in Infected Persons

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Plasma viremia during HIV-1 infection is regulated by a dynamic balance between viral replication and removal of infected cells and cell-free virus. Administration of novel potent antiretroviral drugs provides an opportunity to study the consequences of perturbing this equilibrium by blocking *de novo* infections. In this study, we examined the expression of differentially spliced forms of HIV-1 mRNA, unspliced (US) and multiply spliced (MS), in peripheral blood mononuclear cells (PBMCs) of patients treated with HIV protease inhibitors or combination therapy. In all nine patients studied, a significant reduction in the MS/US mRNA ratio was observed after 1 week of treatment, suggesting that the majority of HIV MS mRNA in the steady-state situation prior to therapy was expressed by cells which had been infected during the previous couple of days. This idea was supported by a detailed analysis of serial PBMC specimens collected from two of the patients during the first hours and days after initiation of therapy. In both cases, a substantial decrease in MS mRNA expression was evident already after 48 hr, whereas the expression of US mRNA at this time was virtually unaffected. These data indicate that the HIV mRNA splicing pattern *in vivo* is mainly determined by the relative proportion of newly infected cells and suggest that examination of this pattern could be useful in evaluating the potency of antiretroviral therapies and in studying dynamics of HIV-1 infection. © 1997 Academic Press

INTRODUCTION

Differential splicing of HIV-1 RNA generates more than 30 distinct mono- or bicistronic mRNA species which fall into three classes: the unspliced (US) full-length 9-kb mRNA, partially spliced 4- to 5-kb mRNAs, and the multiply spliced (MS) ~2-kb mRNAs (Neumann *et al.*, 1994; Schwartz *et al.*, 1990, 1992; Pavlakis *et al.*, 1991). US mRNA encodes the Gag and Gag-Pol polyproteins, and also serves as the genomic RNA of progeny virions. MS mRNAs give rise to the regulatory and accessory HIV proteins Tat, Rev, Nef, Vpr, and Vif.

Kinetic analyses of HIV mRNA expression *in vitro* in newly infected cells (Kim *et al.*, 1989; Klotman *et al.*, 1991; Ranki *et al.*, 1994) and in activated latently infected cells (Michael *et al.*, 1991; Pomerantz *et al.*, 1990) have revealed a temporal pattern of mRNA splicing consisting of early expression of mostly MS mRNA which changes during the subsequent 12–48 hr to a predominance of US mRNA. This process has been attributed to increasing levels of the Rev protein which binds to US and partially spliced mRNAs containing a Rev-responsive element and is required for efficient accumulation of these mRNAs in the cytoplasm of infected cells (reviewed in

Cullen, 1994; Pavlakis *et al.*, 1991; Pavlakis and Felber, 1990).

HIV-1 mRNA splicing has also been studied *in vivo* using reverse transcription-initiated polymerase chain reaction (RT-PCR) techniques. Intriguingly, an early study reported a dramatic overexpression of MS RNA relative to US mRNA in peripheral blood mononuclear cells (PBMCs) of most asymptomatic (but not symptomatic) subjects, suggesting that clinical latency of HIV infection is associated with a "blocked early-stage" HIV mRNA splicing pattern, as if the viral life-cycle in these cells was arrested in an early postintegration step (Seshamma *et al.*, 1992). Although similar results have not been observed in other studies, some recent reports have also suggested that HIV-1 mRNA splicing pattern in PBMC could be of clinical significance, and indicated an association between a reduced MS/US ratio and a poor prognosis (Furtado *et al.*, 1995; Michael *et al.*, 1995).

Our longitudinal studies on a large HIV-1-infected cohort have not provided support for this hypothesis. We found that while the overall HIV mRNA expression strongly correlated with prognosis, MS and US mRNAs were expressed in roughly equimolar ratios in PBMCs of most asymptomatic individuals regardless of the subsequent course of their disease (Saksela *et al.*, 1994, 1995; Vesanen *et al.*, 1996). Similar findings were recently reported by Pavlakis and colleagues based on their detailed examination of a large panel of individual PBMC HIV-1 mRNA species during disease progression (Saltar-

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elli *et al.*, 1996). Furthermore, in cases when we have found the MS/US ratio to significantly deviate from 1:1, no association with a particular future disease pattern has been observed. Similar to the studies by Michael *et al.* and Furtado *et al.*, however, we (Saksela *et al.*, 1994; Vesanen *et al.*, 1996) and others (Saltarelli *et al.*, 1996) have noticed a relative loss of MS mRNA in PBMCs of some individuals upon development of CD4+ T lymphopenia. The underlying causes of such aberrant HIV mRNA splicing patterns are yet to be elucidated.

Recent studies on patients treated with novel potent antiretroviral drugs have shown that the viral load in plasma of infected individuals is determined by a highly dynamic equilibrium between HIV production and clearance (Ho *et al.*, 1995; Wei *et al.*, 1995). Moreover, it has been shown that the rapid decline in plasma viremia results from the decay of two components: removal of productively infected cells ($T_{1/2} \sim 1.6$ days) and clearance of cell-free virus ($T_{1/2} \sim 6$ hr) (Perelson *et al.*, 1996). Thus, it can be concluded that approximately half of the productively infected PBMCs at any given time have become infected as recently as during the preceding 2 days.

Therefore, assuming that the temporal changes in HIV mRNA splicing pattern that occur *in vivo* are similar to those seen *in vitro* (Kim *et al.*, 1989; Klotman *et al.*, 1991; Michael *et al.*, 1991; Pomerantz *et al.*, 1990; Ranki *et al.*, 1994), it is predicted that in a steady-state situation, expression of viral MS mRNA relative to US mRNA would be determined by the proportion of recently infected cells among all circulating productively infected cells, and that blocking infection of new cells by potent antivirals should result in a rapid decrease in the MS/US mRNA ratio. The present results provide experimental data to directly support this scenario, thereby further substantiating the notion of a rapid turnover of productively infected cells *in vivo*, and clarifying the constitution of HIV-1 mRNA splicing patterns observed in HIV-infected persons.

MATERIALS AND METHODS

Nucleic acid preparation

Total cellular RNA and DNA were extracted from PBMC specimens using TRI Reagent (Molecular Research Center, Inc., Cincinnati, OH) according to the instructions from the supplier. Additionally, isolated RNA preparations were treated for 1 hr with 100 U of RNase-free DNase (Boehringer Mannheim) in the presence of 50 U of placental RNase inhibitor (Boehringer Mannheim), extracted with phenol-chloroform, and precipitated with ethanol.

cDNA synthesis

Two micrograms of total DNase-treated PBMC RNA in 20 μ l of water was heated for 5 min at 80°, transferred

onto ice, and combined with a mixture containing: 6 μ l 5 \times reaction buffer (5 \times : 250 mM Tris, pH 8.3; 375 mM KCl, 15 mM MgCl₂), 1.5 μ l dNTPs 10 mM each, 0.6 μ l RNasin, 1 μ l random hexamers (1 mM, Pharmacia), and 2 μ l M-MLV-reverse transcriptase (200U/ μ l; Gibco-BRL) and incubated for 1 hr at 42°.

Amplification of US HIV-1 mRNA

Two-microliters of random-primed PBMC cDNA was added into each 50 μ l PCR reaction containing 10 mM Tris, pH 8.5, 50 mM KCl, 1.0 mM MgCl₂, 0.02% gelatin, 50 μ M of each unlabeled dNTPs, 150 ng each oligonucleotide primer, 0.25 μ l α [³²P]dCTP (NEN, 3000 Ci/mmol), and 0.25 μ l Taq-polymerase (5 U/ μ l; Boehringer Mannheim). These reactions were cycled for 31 rounds between 94 (40 sec) and 69° (1 min) using Perkin-Elmer GeneAmp PCR system 9600. The primers used for US HIV-1 mRNA amplification were: 5'-TCT CTA GCA GTG GCG CCC GAA CA-3' (sense) and 5'-TCT CCT TCT AGC CTC CGC TAG TC-3' (antisense) giving rise to a 160-bp product. This assay detects full-length HIV-1 mRNA encoding Gag and Gag-Pol proteins as well as genomic HIV RNA.

Amplification of MS HIV-1 mRNA

MS HIV-1 mRNA was amplified using a nested PCR strategy. Both rounds of amplification were done using the same reaction conditions as described for US mRNA and utilized a common antisense primer: 5'-TTC CTT CGG GCC TGT CGG GTC GG-3'. The sequence of the outer sense MS primer was 5'-CTT AGG CAT CTC CTA TGG CAG GAA-3', and the inner one was 5'-TCC TAT GGC AGG AAG AAG CGG AG-3' (resulting in fragments of 131 and 121 bp). After the first round of amplification consisting of 25 cycles, 5 μ l of this reaction was added into triplicate nested reactions which were cycled 11, 17, or 22 rounds. The secondary reaction in which the signal was in the optimal linear range of amplification (as compared to a standard dilution curve) was subsequently used to estimate MS HIV mRNA copy-number in the specimen (see below). This assay detects small HIV-1 mRNAs giving rise to Tat, Vif, Vpr, Rev, and Nef proteins.

Amplification of GAPDH mRNA

To monitor the recovery of intact PBMC RNA from different PBMC specimens and the uniform efficiency of each reverse transcription reaction, a 189-bp glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA-specific fragment was also amplified from each random-primed cDNA preparation. These reactions were cycled for 20 rounds between 94 (1 min) and 67° (2 min) with conditions otherwise as described above, and utilized the primers 5'-TGG TAT CGT GGA AGG ACT CAT GAC-3' (sense) and 5'-ATG CCA GTG AGC TTC CCG TTC AGC-3' (antisense).

Controls, RNA standards and quantitation of amplification products

Each set of reverse transcription reactions included a control tube with no RNA which was subsequently used for US- and MS-specific PCR reactions as a control for lack of contamination. In addition, every set of experiments included a control RNA from PBMCs of an HIV-negative donor. To provide standards for quantitation of the absolute HIV mRNA copy-numbers in the specimens, all experiments also included a panel of 10 PBMC RNAs with twofold serial dilutions of *in vitro*-transcribed US and MS RNA templates (Saksela *et al.*, 1994) ranging from 64 to 100,000 copies/ μ g of HIV-negative PBMC RNA. Ten microliters of each PCR reaction was analyzed by electrophoresis in 8% acrylamide:bisacrylamide (19:1) gels in 1 \times TBE (90 mM Tris-borate, 1 mM EDTA), and the intensities of the radiolabeled amplification products in the dried gels were quantitated using a PhosphorImager (Molecular Dynamics, Inc., Sunnyvale, CA). The specific signals corresponding to the US and MS HIV mRNA-specific fragments (the value from an identical area above the specific amplification signal in each lane was subtracted) were normalized based on the specific GAPDH amplification signals from the same cDNA preparations, and used to calculate the absolute HIV mRNA copy-numbers in the specimens by comparing these signals with those of the HIV RNA standards analyzed in parallel. In addition, every assay included an aliquot of a common HIV-infected PBMC preparation. The interassay variation in the derived mRNA copy-numbers from repeated analyses of this specimen was less than 25%. Threshold of linear amplification of unspliced (US) and multiply spliced (MS) HIV-1 mRNAs is \sim 100 copies per microgram of total PBMC RNA.

RESULTS

Paired PBMC samples collected before and 1 week after treatment with a HIV protease inhibitor or combination therapy were selected from nine patients involved in published and ongoing studies on dynamics of HIV-1 infection and efficacy of antiviral therapies (Perelson *et al.*, 1996; Table 1). Total RNA was extracted from PBMCs and subjected to quantitative RT-PCR to measure the expression of US and MS mRNAs as described under Materials and Methods. Figure 1 shows the absolute copy-numbers of US and MS mRNAs (per μ g of PBMC RNA) in these specimens, and also displays as a bar diagram the ratio of MS/US mRNA in each patient before and after 7 days of drug therapy. As can be seen, regardless of the overall level of HIV mRNA expression, in each case a significant change in MS/US ratio was observed, such that this ratio decreased on average to 34% of its pretreatment value. These results suggest that a change in HIV mRNA splicing pattern is a consistent consequence of potent antiretroviral drug therapy and agree

well with our earlier observations on some AZT-treated patients (Saksela *et al.*, 1994) as well as with data recently reported by Bagnarelli *et al.* (1996).

The observed change in MS/US ratio was caused by the considerably smaller reduction in the expression of US mRNA as compared to MS mRNA. In fact, in some patients little or no decrease in US mRNA was observed at 1 week after treatment, although a significant drop in plasma viremia was evident in all patients at this point (Table 1). This observation may reflect the trafficking of lymphocytes (infected or not) from tissues into the circulation or could also be due to HIV genomic RNA associated with uninfected or nonproductively infected PBMCs (see Discussion).

A week of effective antiretroviral therapy (Table 1) can be expected to cause a significant decrease in the relative number of cells which have been infected during the preceding few days. In light of available kinetic data from studies in cell culture, the observed shift in HIV mRNA splicing pattern after therapy therefore suggests that in a steady-state situation, this pattern directly reflects the proportion of cells infected at various times before the analysis. The change in the HIV mRNA splicing pattern from the early MS mRNA-predominated pattern to the subsequent relative overexpression of US mRNA takes place within 12–48 hr of infection *in vitro*. To examine the kinetics of the splicing shift induced by antiretroviral therapy *in vivo*, HIV mRNA expression after initiation of drug therapy was analyzed in greater detail in two patients (105 and 1302) from whom frequently collected PBMC specimens were available.

Expression of HIV US and MS mRNA in PBMCs collected from these two patients before treatment and at every 6–12 hr during the first 3 days of therapy is shown in Fig. 2. In both cases, a substantial decrease in the expression of MS mRNA was observed during this period, with much of this reduction occurring between 12 and 48 hr after initiation of therapy (from 4271/ μ g to 651/ μ g in 105, and from 2701/ μ g to 823/ μ g in 1302). By contrast, no apparent decrease in HIV US mRNA was observed during this period. These results are in good agreement with kinetic data on HIV mRNA splicing during viral life-cycle *in vitro* (Kim *et al.*, 1989; Klotman *et al.*, 1991; Michael *et al.*, 1991; Pomerantz *et al.*, 1990) and thus suggest that the marked reduction in HIV MS mRNA expression *in vivo* following administration of potent antiviral drugs is due to a rapidly developing loss of cells in an early postintegration phase of HIV life-cycle.

DISCUSSION

The availability of novel potent antiretroviral drugs have enabled us to perform an analysis of HIV mRNA expression akin to an *in vivo* "pulse-chase experiment." As predicted by *in vitro* studies on the regulation of HIV mRNA splicing during viral life-cycle (Kim *et al.*, 1989;

TABLE 1

Summary of the Treatment Modality, Base-Line Values, and the Effect of 7 Days of Therapy on Plasma Viral Load of the Study Patients

Patient	Treatment/total daily dose	Base-line values		
		CD4 cells (/mm ³)	Plasma virions (×10 ³ /ml) ^a	Plasma virions after 7 days of therapy (×10 ³ /ml) ^a
2-3	Nelfinavir/1000 mg	269	50	2.5
2-18	Nelfinavir/3000 mg	334	64	5.9
2-26	Nelfinavir/3000 mg	458	58	8.1
1302	Indinavir, 3TC, AZT/2400 mg, 300 mg, 600 mg	227	3166	52
102	Ritonavir/1200 mg	16	294	55
103	Ritonavir/1200 mg	408	12	0.7
104	Ritonavir/1200 mg	2	52	8.0
105	Ritonavir/1200 mg	11	643	85
107	Ritonavir/1200 mg	412	77	8.0

^a Each virion contains two RNA copies.

Klotman *et al.*, 1991; Michael *et al.*, 1991; Pomerantz *et al.*, 1990; Ranki *et al.*, 1994), as well as recent insights into the dynamics of HIV infection *in vivo* (Ho *et al.*, 1995; Perelson *et al.*, 1996; Wei *et al.*, 1995), a rapid change in HIV mRNA splicing was observed in the drug-treated patients. These data suggest that the early loss in MS mRNA is caused by a change from an early-stage to a

late-stage splicing pattern due to the halt of new PBMCs entering the pool of productively infected cells.

Curiously, in some patients (such as 105) US HIV mRNA decreased slower than what one would expect based on the drop in their plasma viral loads. As mentioned above, it could be that upon decreasing viral load in the body, more lymphocytes (including infected cells

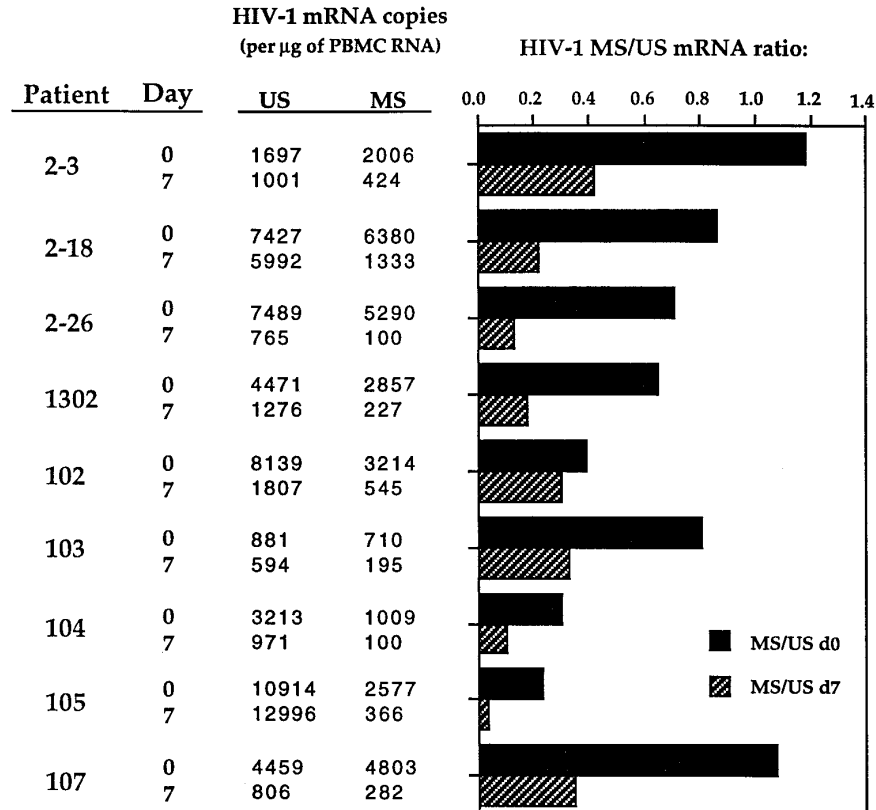


FIG. 1. The absolute copy numbers of HIV-1 unspliced (US) and multiply spliced (MS) mRNAs (per microgram of total PBMC RNA) and a bar diagram of their ratio before and 7 days after the therapy in each of the nine study subjects.

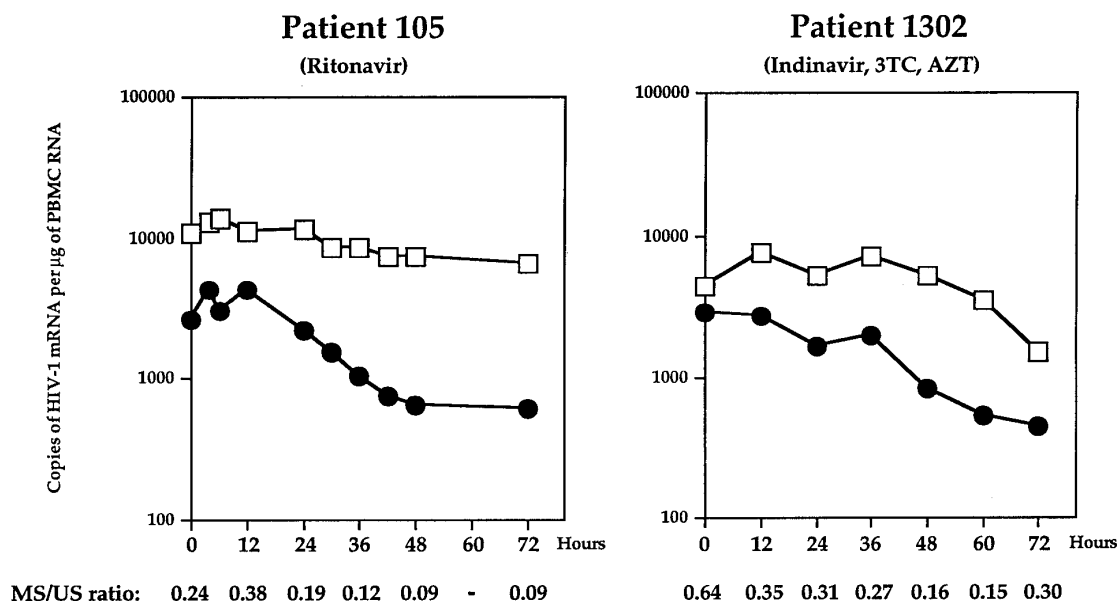


FIG. 2. Dynamics of HIV-1 splicing pattern in two study subjects during the first 3 days of therapy. Their PBMC HIV-1 unspliced (US; *ii/home2/milesgrsym/8point/g170,l(3,0) and multiply spliced ii/home2/milesgrsym/8point/g172,l(3,0) mRNA expression is shown as copies per microgram of total cellular RNA on a log scale. The MS/US mRNA ratio is presented at 12-hr intervals. (–) Not detected.

with a late-stage mRNA splicing pattern) enter the circulation thus providing an additional source of US mRNA. However, an alternative explanation for this observation could be that the decay of US mRNA might be partially masked by the presence of genomic HIV RNA in the PBMC RNA preparations. Such "treatment-resistant" genomic RNA could be derived from viral particles stuck to the surface of PBMCs and survived through the fractionation and washing of these cells. More interestingly (and perhaps more likely) significant numbers of HIV genomes might be present in resting T cells as preintegration complexes frozen in the viral life-cycle between entry and integration (Bukrinsky *et al.*, 1991; Chun *et al.*, 1995; Stevenson *et al.*, 1990; Zack *et al.*, 1990). Although such "preintegration latency" is probably relatively short-lived, it could give rise to an artifactually slow decay rate of US mRNA in the present analysis. However, such unaccounted sources of US HIV mRNA signal (if they exist in significant amounts) are not likely to challenge the present conclusions which are based on the prominent fall in MS HIV mRNA levels occurring during the first 48 hr of therapy without any accompanying decrease in the expression of US HIV mRNA.

Besides supporting the current dynamic model of HIV infection (Ho *et al.*, 1995; Perelson *et al.*, 1996; Wei *et al.*, 1995), the present results suggest that HIV mRNA splicing in PBMCs *in vivo* is mainly determined by the proportion of cells in different phases of infection. Situations apart from antiretroviral therapy that could result in changes in the ratio of MS and US mRNAs in HIV-infected persons can be envisioned. For example, transient activation of viral replication by environmental or other stim-

uli, as reported after influenza vaccination (O'Brien *et al.*, 1995; Staprans *et al.*, 1995) or IL-2 administration (Kovacs *et al.*, 1995) could result in an increase of cells which are in an early phase of infection, manifesting as a relative overexpression of MS mRNA. On the other hand, it is possible that when HIV infection is spreading relatively unimpeded, as might be the case in severely immunodeficient patients, many more susceptible CD4+ cells are already infected, resulting in a fewer number of newly infected cells and thus a relative overexpression of US mRNA. However, although such factors could cause alterations in the HIV mRNA splicing pattern, our data do not support a hypothesis that differential splicing would be an actively regulated process involved in determining the productivity of HIV-infection and the rate of disease progression.

The present results suggest that examination of HIV MS/US mRNA ratio as an indicator of population dynamics of HIV-infected cells *in vivo* could be useful. By providing an almost instantaneous measure of the acute antiviral potency of a drug regimen, monitoring of the early decrease in MS HIV mRNA expression might be used as an additional molecular virological tool in developing and optimizing pharmacological agents to block HIV infection. In addition, more detailed analyses of the expression of differentially spliced HIV mRNAs in PBMCs and other sites of HIV replication in the body might offer further insights into the dynamics of HIV infection *in vivo*.

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